Late-Phase Contrast-Enhanced Ultrasound Reflects Biological Features of Instability in Human Carotid Atherosclerosis

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Background and Purpose—Development of translational functional imaging modalities for atherosclerosis risk stratification is sought for stroke prediction. Our group has developed late-phase contrast-enhanced ultrasound (LP-CEUS) to quantify microbubble contrast retention within carotid atherosclerosis and shown it to separate asymptomatic plaques from those responsible for recent cerebrovascular events. We hypothesized that microbubbles are retained in areas of plaque inflammation, aiming to examine whether LP-CEUS signal reflects plaque biology.

Methods—Subjects awaiting carotid endarterectomy (n=31) underwent axial LP-CEUS and diseased intimal segments were symmetrically divided in the long axis. Half-specimens underwent quantitative immunohistochemical analysis for CD68 (macrophages) and CD31 (angiogenesis). Half-specimens were processed for atheroma cell culture and supernatant collected at 24 hours for multianalyte profiling for 34 analytes.

Results—Percentage area immunopositivity was significantly higher in subjects in which normalized plaque late-phase intensity was ≥0 versus <0 (CD68 mean 11.8 versus 6.68, P=0.004; CD31 mean 9.45 versus 4.82, P=0.025). Interleukin-6, matrix metalloproteinase-1, and matrix metalloproteinase-3 were significantly higher by multianalyte profiling when LP-CEUS was ≥0.

Conclusions—LP-CEUS reflects biological features of inflammation and angiogenesis, key features predisposing to plaque rupture. Further investigation of LP-CEUS as a tissue-specific marker of inflammation for risk stratification of carotid atherosclerosis is warranted. (Stroke. 2011;42:3634-3636.)

Key Words: angiogenesis □ atherosclerosis □ inflammation □ late-phase contrast-enhanced ultrasound □ stroke

Stroke continues to have a significant personal and health-economic impact. Atherosclerosis of the carotid bifurcation is responsible for approximately one fifth of these cerebrovascular events, in which inflammation, angiogenesis, and matrix degradation are key determinants of plaque vulnerability.1 Functional imaging modalities are being developed to visualize these biological features in vivo.

Contrast-enhanced ultrasound (CEUS), in its dynamic phase (immediately postcontrast), can assess carotid vasa vasorum, relating findings to histological evaluation of angiogenesis and cardiovascular disease and past cardiovascular events.2 Recently, late-phase- (LP-) CEUS at 6 minutes postcontrast has distinguished human symptomatic from asymptomatic carotid plaques in vivo.3 In this study, our primary hypothesis was that LP-CEUS would reflect inflammation and angiogenesis determined by immunohistochemistry. We further wanted to explore whether LP-CEUS could offer information about cytokine, chemokine, matrix metalloproteinase (MMP), and tissue inhibitor of metalloproteinase levels.

Methods
Research ethics and Medicines and Healthcare Products Regulatory Agency approvals were granted. Consecutive consenting subjects awaiting carotid endarterectomy were recruited. We excluded subjects experiencing myocardial infarction or unstable angina within 14 days of study commencement and those with New York Heart Association III or IV heart failure or prosthetic heart valves (contraindications to contrast administration).

Thirty-one subjects were enrolled (mean age, 70.5 years; 24 males). LP-CEUS signal from a number of these patients has previously been reported.3 Carotid plaques from 16 (52%) patients were symptomatic if stroke, transient ischemic attack, or amaurosis fugax occurred in the index plaque neurovascular territory within 12 months of enrollment. Median time from symptoms to LP-CEUS was 21.0 days.

Late-Phase Contrast-Enhanced Ultrasound
Carotid LP-CEUS was performed with a Philips iU22 (Bothel, WA) scanner, L12-5 MHz probe, and 2 mL of SonoVue (Bracco, Italy). Axial flash-imaging (mechanical index 0.34) in nonlinear (power modulation) contrast mode at 6 minutes postbolus at the level of greatest stenosis was as previously described.3 Quantification of mean plaque and residual luminal echo intensities used QLAB.
Raw linear data were log transformed with plaque signal normalization against luminal signal. Receiver operating characteristic curve analysis showed a normalized LP-CEUS signal of 0 was optimum to distinguish symptomatic from asymptomatic plaques; hence, subjects were grouped according to a signal cutoff of 0.

Fresh endarterectomy specimens were divided symmetrically along their long axis, allowing undertaking of immunohistochemistry and atheroma cell culture (n=19). Where there was insufficient material, specimens were assigned to either histology (n=10) or atheroma cell culture (n=2) alone.

**Immunohistochemistry**

Atheroma specimens (n=29) were axially divided into 3, representing proximal, middle, and distal plaque regions. Three 7-μm cryosections from each of the 3 regions were immunohistochemically stained for the macrophage marker CD68 (PG-M1, 1:500; Dako) and the endothelial cell marker CD31 (JC70A, 1:1000; Dako). Semiautomated image analysis (Vision 5.0; Clemex) quantified average percentage area immunopositivity across the 9 sections for both CD68 and CD31.

**Atheroma Cell Culture and Multianalyte Profiling**

Fresh plaque segments (n=21) were enzymatically digested in a collagenase/elastase/DNAse mixture by a validated methodology. Mixed cell suspensions obtained were cultured at 1×10⁶ cells/mL in RPMI containing 10% fetal bovine serum (Biosera). Supernatants collected at 24 hours were stored at −80°C for single-batch analysis. Multianalyte profiling using Luminex 100 quantified supernatant protein levels of 18 cytokines and 4 chemokines (Milliplex; Millipore Corporation), 8 MMPs and 4 tissue inhibitor of metalloproteinas (Fluorokine; R&D Systems) in duplicate. Where an analyte level was below detection limits, it was ascribed the lowest standard value for statistical analysis.

**Results**

Fifteen (48%) subjects had LP-CEUS signal <0 and 16 (52%) ≥0. Groups were matched for degree of carotid stenosis, demographic and clinical parameters, statin use, and plasma C-reactive protein. Both groups had a median 1-day interval between LP-CEUS and endarterectomy. There were significantly more symptomatic stenoses in the LP-CEUS ≥0 group (P=0.001).

CD68 and CD31 immunopositivity were significantly higher in subjects in which LP-CEUS was ≥0 versus <0 (Figures 1 and 2). There was a significant positive correlation between LP-CEUS and CD68 immunopositivity (n=29, r=0.466, P=0.011). Multianalyte profiling detected 11 of 18...
cytokines, 4 of 4 chemokines, 7 of 8 MMPs, and 4 of 4 tissue inhibitor of metalloproteinases. Interleukin-6, MMP-1, and MMP-3 were significantly higher in the LP-CEUS versus the group (Figure 3).

Discussion
Our results show human atherosclerotic plaques with normalized LP-CEUS signal ≥0 have significantly more inflammation (CD68, interleukin-6), angiogenesis (CD31), and matrix degradation (MMP-1, MMP-3) than plaques with signal <0; these are key biological features involved in the initiation, progression, and complications of atherosclerosis.1

This clinical study supports preclinical investigation showing nontargeted microbubbles are passively retained within tissue where there is inflammation and/or endothelial activation.5,6 It is hypothesized that microbubbles adhere to endothelium or leave microvessels, entering plaque parenchyma, retained in isolation, or within phagocytosing macrophages.

The limitations of our work include the numbers of subjects and the potential for sampling error. The latter may be overcome using 3- or 4-dimensional volumetric ultrasound acquisition under investigation by our group; however, spatial resolution is presently not adequate for use in LP-CEUS.

Conclusions
This study demonstrates LP-CEUS reflects biological features of inflammation and angiogenesis, which are key to plaque rupture. Furthermore, exploratory analysis suggests that LP-CEUS may offer information about intraplaque matrix degradation. These findings support further study of LP-CEUS as a tissue-specific marker of inflammation with potential use in carotid risk stratification.

Sources of Funding

Disclosures
T.G. is an employee of Phillips.

References
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Stroke. 2011;42:3634-3636; originally published online September 29, 2011; doi: 10.1161/STROKEAHA.111.631200

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/42/12/3634

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